

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 October 2001 (04.10.2001)

PCT

(10) International Publication Number  
**WO 01/73094 A2**

(51) International Patent Classification<sup>7</sup>: C12N 15/87,  
5/10, A01K 67/027, C07K 16/44

(21) International Application Number: PCT/US01/07018

(22) International Filing Date: 5 March 2001 (05.03.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
09/537,861 28 March 2000 (28.03.2000) US

(71) Applicant: BIOAGRI CORPORATION [US/US];  
17711 Rowland Street, City of Industry, CA 91748 (US).

(72) Inventor: WANG, Kangsheng; 2125 South Edmore Avenue,  
Rowland Heights, CA 91748 (US).

(74) Agent: WISE, Michael, J.; Lyon & Lyon LLP, 633 West  
Fifth Street, Suite 4700, Los Angeles, CA 90071-2066  
(US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM,  
HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK,  
LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX,  
MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL,  
TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

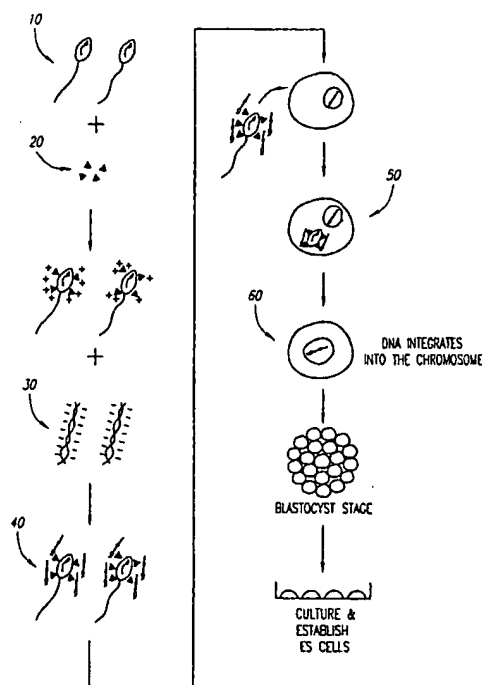
(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished  
upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance  
Notes on Codes and Abbreviations" appearing at the beginning  
of each regular issue of the PCT Gazette.*

(54) Title: A NEW VECTOR FOR GENETICALLY MODIFYING NON-HUMAN ANIMALS



(57) Abstract: The present invention is directed to a vector and its use to generate genetically modified animals and cells. One aspect of this invention involves a vector that comprises a sperm cell and one or more polynucleotide molecules bound to a sperm cell through one or more non-liposome based linkers. The sperm cell can be any animal sperm cell, preferably non-human animal. In one preferred embodiment of this invention, the one or more polynucleotide molecules encode for a gene product that confers desired characteristics in the cells or the animals. In another preferred embodiment of this invention, the linker is a protein or polypeptide, preferably sperm specific such as an antibody that binds with the external surface of the sperm cell. The linker interacts with one or more polynucleotide molecules preferably by ionic interaction. This interaction can also be carried out by different molecular interactions, including the use of another or secondary linker. The association of the sperm, linker, and the one or more polynucleotide can also occur *in vitro* or *in vivo*. In another aspect of the present invention genetically modified cells or animals are derived from the fertilization of an animal egg cell with the vector described above. Fertilization can occur *in vitro* or *in vivo*. In one preferred embodiment, genetic modification occurs with the polynucleotide molecule integrating, wholly or partially, into the cell or animal's genome. Another aspect of the present invention includes cells, such as sperm cells or egg cells, and cell lines that are derived from these genetically modified animals or their descendants. In another aspect of the present invention, the genetically modified animals derived from the use of the sperm vector described above possess certain desired

characteristics. Examples of these characteristics include faster growth rates, disease or pathogen resistance, high production of certain proteins in milk, and organs suitable for animal to human xenotransplantation.

## DESCRIPTION

### A New Vector For Genetically Modifying Non-Human Animals

#### Field Of Invention

5           The present invention relates to the field of genetic modification in non-human animals.

#### Background Of The Invention

Efficient genetic modification of animals, especially in higher mammals, has been a major goal of researchers in the biotechnology field for the last two decades. Not only  
10       can genetic modification of animals advance our understanding of genes and gene-functions in multi-cell organisms, it can also serve useful applications in the bio-agricultural industry. Examples of these applications include raising livestock with desired characteristics such as faster growth rate, production of therapeutic proteins in milk, or even the generation of more "humanized" organs from animals for use in animal  
15       to human xenotransplantation.

Current techniques to modify the genome include microinjection of foreign DNA into the pronuclei of fertilized eggs, delivery of foreign DNA into embryonic stem cells *in vitro* or blastomere cells *in vivo* through lipid-based agents, electroporation, or viral infection. Aside from mice, however, current techniques have been reported to have had  
20       limited success in higher or larger animals. The microinjection technique, for example, has been reported to be technically very demanding and requires the use of highly sensitive and expensive equipment. The viability of embryos after microinjection has also been reported to be very poor. Wall, R.J., et. al. (1992) Making Transgenic Livestock, Genetic Engineering on a Large Scale, *Journal of Cellular Biochemistry*, Vol. 49, pp. 113-  
25       120. This has led researchers in the field to investigate alternative and easier ways of delivering genes into an animal.

In 1989, Lavitrano, M., et. al. reported that simply incubating foreign DNA with mice's sperm cells and effecting fertilization *in vitro* could lead to genetically modified mice. Lavitrano, M., et. al. (1989) Sperm Cells as Vectors for Introducing Foreign DNA  
30       into Eggs - Genetic Transformation of Mice, *Cell*, Vol. 57, pp. 717-723. Characterized as the "cold fusion" equivalent in biotechnology, this report generated much excitement in the field. Birnstiel, M., et. al. (1989) Dangerous Liaisons: Spermatozoa as Natural Vectors for Foreign DNA?, *Cell*, Vol. 57, pp. 701-702. Those skilled in the art, however, are reported to remain skeptical even to this day about the Lavitrano's report since a number

of researchers in the field have reportedly failed to repeat the experiment. Brinster, R., et. al. (1989) No Simple Solution for Making Transgenic Mice, *Cell*, Vol. 59, pp. 239-241; Smith, K. (1999) Sperm Cell Mediated Transgenesis: A Review, *Animal Biotechnology*, Vol. 10(1&2), pp. 1-13.

- 5 Over the last decade, efforts have continued to explore the use of sperm cells as a vector for mediating gene transfer in animals. Researchers have elucidated that sperm cells have the inherent ability to internalize foreign DNA. Francolini, M., et. al (1993) Evidence for Nuclear Internalization of Exogenous DNA into Mammalian Sperm Cells, *Mol. Reprod. Devel.*, Vol. 34, pp. 133-139. Yet, certain inhibitory factors present in
- 10 seminal fluid may inhibit this ability to take up DNA. Lavitrano, M., et. al. (1992) The Interaction Between Exogenous DNA and Sperm Cells, *Mol. Reprod. Devel.*, Vol. 31, pp. 161-169. In addition, foreign DNA introduced into sperm cells may also suffer from extensive DNA rearrangement because in mature sperm cells, internalization of foreign DNA may activate certain endogenous nucleases in these cells. Maione, B. et. al. (1997)
- 15 Activation of Endogenous Nucleases in Mature Sperm Cells upon Interaction with Exogenous DNA, *DNA and Cell Biology*, Vol. 16, pp. 1087-1097. Such rearrangement could threaten the usefulness of genetically modified animals using this technique.

- Other work with sperm cells as vector have focused on the use of either lipid-based agents or electroporation to deliver foreign DNA into the sperm cells. Smith, *supra*;
- 20 Rottman R., et. al. (1996) Liposome-mediated Gene Transfer via Sperm Cells. High Transfer Efficiency and Persistence of Transgenes by Use of Liposomes and Sperm Cells and a Murine Amplification Element, *Journal of Animal Breeding and Genetics*, Vol. 113, pp. 401-411; PCT Publications WO 99/42569, WO 99/40213, and WO 97/11597. Such methods may also suffer from the same problem of DNA internalization and exposure to
- 25 nucleases that could cause rearrangement of the foreign DNA being introduced. In addition, lipid-based agents, which are often toxic, and electroporation may require extensive experimentation to prevent the death or the loss of sperm cell motility. Other techniques have also focused on using recombinant virus infection, as disclosed in PCT Publications WO 99/38991, or on using a "gene gun" with micro-carriers, as disclosed in
- 30 PCT Publication WO 93/24626, to introduce foreign DNA into sperm cells. Such techniques may be technically challenging and may also affect the viability and motility of the sperm cells. They may also suffer from the same problem of DNA internalization and exposure to nucleases that could cause rearrangement of the foreign DNA being introduced.

- 35 Since 1989, researchers have reported the use of sperm cells as vectors in different animals ranging from insects, marine animals, amphibians, birds, and mammals. Smith,

*supra*. However, few reported that the genetic modification was observed in viable mature offspring. Smith, *supra*. More problematic is the fact that some reports used only PCR analysis to verify the existence of the foreign DNA in the cells. These reports are summarized in table one of Gandolfi, F. (1998) Spermatozoa, DNA Binding and Transgenic Animals, *Transgenic Research*, Vol. 7, pp. 147-155. Since PCR cannot distinguish between foreign DNA transmitted through episomes or through the chromosomal DNA, Gandolfi has questioned the value of these reports stating that it "opens up an important argument relating to appropriate evaluation of the results described in some reports." Gandolfi, *supra*. Episomal transmission is not as desirable as chromosomal transmission since the episome may be lost during subsequent cell division, and the desired effect of genetic modification may never be expressed in adult animals.

Because an easy, non-toxic, and efficient way of genetically modifying animals, especially in higher mammals, can greatly advance this field, a new way of using sperm cells for delivering genes into animals is needed.

#### 15 Summary Of The Invention

The present invention is directed to a vector and its use to generate genetically modified animals and cells. One aspect of this invention involves a vector that comprises a sperm cell and one or more polynucleotide molecules bound to a sperm cell through one or more non-liposome based linkers. The sperm cell can be any animal sperm cell, preferably non-human animal. In one preferred embodiment of this invention, the one or more polynucleotide molecules encode for a gene product that confers desired characteristics in the cells or the animals. In another preferred embodiment of this invention, the linker is a protein or polypeptide, preferably a sperm specific linker that binds with the external surface of the sperm cell. The linker interacts with one or more polynucleotide molecules preferably by ionic interaction. This interaction can also be carried out by different molecular interactions, including the use of another or secondary linker. The association of the sperm, linker, and the one or more polynucleotide can also occur *in vitro* or *in vivo*.

In another aspect of the present invention, genetically modified cells or animals are derived from the fertilization of an animal egg cell with the vector described above. Fertilization can occur *in vitro* or *in vivo*. In one preferred embodiment, genetic modification occurs with the polynucleotide molecule integrating, wholly or partially, into the cell or animal's genome. Another aspect of the present invention includes cells, such as sperm cells or egg cells, and cell lines that are derived from these genetically modified animals or their descendants.

In another aspect of the present invention, the genetically modified animals derived from the use of the sperm vector described above possess certain desired characteristics. Examples of these characteristics include faster growth rates, disease or pathogen resistance, high production of certain proteins in milk, and organs suitable for animal to human xenotransplantation.

#### Brief Description Of The Drawings

Figure 1 is a pictorial representation of the basic steps involved in using one embodiment of the present invention.

Figure 2 shows a flow-cytometry result of binding a sperm-specific antibody to mice's sperm cells as embodied in one aspect of the present invention.

Figure 3 shows a flow-cytometry result of binding a sperm-specific antibody to pig's sperm cells as embodied in one aspect of the present invention.

Figure 4 shows a flow-cytometry result of binding a sperm-specific antibody to cow's sperm cells as embodied in one aspect of the present invention.

Figure 5 shows a flow-cytometry result of binding a sperm-specific antibody to chicken's sperm cells as embodied in one aspect of the present invention.

Figure 6 shows a flow-cytometry result of binding a sperm-specific antibody to goat's sperm cells as embodied in one aspect of the present invention.

Figure 7 shows a flow-cytometry result of binding a sperm-specific antibody to sheep's sperm cells as embodied in one aspect of the present invention.

Figure 8 shows a plasmid map of pCMV- $\beta$ .

Figure 9 shows an agarose-gel analysis of a sperm-specific antibody binding to pCMV- $\beta$  plasmid.

Figure 10 show results of PCR analysis for the detection of pCMV- $\beta$  sequences in genomic DNA isolated from mice's embryos genetically modified according to one embodiment of the present invention.

Figure 11 shows results of southern-blot analysis for the detection of Hepatitis B surface-antigen gene-sequence in mice-tail-genomic DNA with this gene-sequence being integrated into the mice's chromosome according to one embodiment of the present invention.

Figure 12 shows the plasmid map of pSEAP-2-control.

Figure 13 shows the result of southern-blot analysis for the detection of pSEAP2-control plasmid sequence in the genomic DNA isolated from tail tissues of genetically modified pigs according to one embodiment of the present invention.

Figure 14 shows the copy number of integrated pSEAP2-control plasmid in four genetically modified pigs based on densitometric intensities of bands in Figure 13.

Figure 15 and 16 show the results of enzyme assays for secreted alkaline phosphatase found in serum of pigs genetically modified according to one embodiment of the present invention.

#### General Description Of The Invention

Generally, figure 1 shows the basic steps involved in using one embodiment of the present invention to genetically modify cells or animals using a sperm vector. Briefly, animal sperm cells 10, are collected by methods known in the art or purchased commercially from sources such as Birchwood Genetics in West Manchester, Ohio, and are bound together with linkers 20. These linkers are preferably antibodies or immunoglobulins of the types, IgG, IgA or IgM, but they can also be other compounds such as peptides, glycoproteins, carbohydrates, or other chemical-compound linkers. These linkers bind or associate to the sperm cells' external surface through different molecular interactions such as ionic interaction, covalent bonds, Vander Waals forces, or ligand-receptor interaction. Circular or linear DNA molecules 30 then bind or attach to the linkers on the sperm-linker complex also through different molecular interactions such as ionic, covalent bonds, Vander Waals forces, or ligand-receptor interaction. These DNA molecules may encode for certain gene products, but they may also be disrupted genes, homologous with endogenous genes, that recombine into the chromosome to knockout a gene. The sperm-linker-DNA complex 40 formed can then be used to effectuate fertilization *in vitro* or *in vivo*. Upon fertilization, the DNA is introduced into the fertilized egg 50 and embryo 60 and can integrate into the chromosome, becoming a part of an animal or cell's genetic material.

Alternatively, the binding, coupling, linking, attaching, or association of the sperm-linker-DNA complex can also be accomplished *in vivo*. The linker and the DNA can first be coupled or bound together *in vitro*. Afterwards, this linker-DNA complex can be injected directly or indirectly into a male animal's testicles. PCT Publications WO 99/40213 and WO 97/11597 disclose procedures for injecting DNA into the testicles, and these publications are incorporated herein by reference.

An example of a linker-DNA complex is an antibody attached with DNA molecules where the antibody specifically recognizes certain surface epitopes on sperm cells. Because of the acidic characteristic of naked DNA, it can ionically associate, bind or, couple with an antibody that has basic or positively charged properties. However, the DNA-linker interaction is not limited to ionic interaction. The complex can also be

crosslinked by UV light to form covalent bonds by well known methods in the art. Both the DNA and the linker can also be modified by methods known in the art. For example, the DNA can be biotinylated by adding biotinylated deoxynucleotides in a PCR reaction; the antibody can be modified or purchased with attached streptavidin, which binds tightly to the biotin on the DNA; or a secondary antibody, which is modified with streptavidin and recognizes the first antibody can also act as a secondary linker between the modified DNA and the first linker.

If the DNA-linker complex is injected into the testis of the animal, this complex can seek out the sperm cells and bind to them. Fertilization can then occur *in vivo* via either natural copulation of the male and female animals or by artificial insemination of the female with collected sperm cells. The collected sperm cells can also be used with *in vitro* fertilization techniques, which are well known in the art. On the other hand, if binding of the sperm-linker-DNA complex, as a whole, occurred *in vitro*, fertilization can be achieved by *in vitro* fertilization techniques. The fertilized eggs and resulting embryos can then be transplanted to surrogate-animal mothers for development. Alternatively, well known artificial insemination methods or injections of the sperm-linker-DNA complex directly into the oviduct of female animals can also achieve fertilization *in vivo*.

Genetically modified animals can serve a number of useful applications. Livestock, poultry, or fish can be inserted with genes that encode for growth hormones to make them grow faster than normal or they can also be inserted with the somatotropin gene to increase muscle growth and decrease adipose tissue. Pursel, V. G., et.al. (1989) Genetic Engineering of Livestock, *Science*, Vol. 244, pp. 1281-1288; Etherton, T.D., et. al. (1993) Mechanism by which Somatotropin Decreases Adipose Tissue Growth, *American Journal of Clinical Nutrition*, Vol. 58 (Supp.), pp. 287S-295S. Inserting genes such as interferon that boost the immune system or other genes, such as genes encoding for viral, prion, or bacterial proteins, can also make these livestock, poultry, or fish disease or pathogen resistant. Examples of these infectious pathogens include Salmonella, influenza virus, prion proteins for the Mad Cow Disease, etc. Alternatively, introducing DNA encoding for anti-sense RNA molecules, which are complementary to these viral, prion, or bacterial RNAs, may also inhibit translation and production of proteins from these RNA, which limits growth and spread of these infectious pathogens.

Moreover, in animals, including insects such as silkworms, that produce raw materials for clothing such as wool and silk, inserting genes for biochemical enzymes that produce the rate-limiting amino acid may increase production of these raw materials. In sheep, for example, the availability of the amino-acid cysteine limits the production of wool. Inserting bacterial genes that encode for serine transacetylase and O-acetylserine



sulfhydrylase may increase the conversion of serine and acetyl-CoA into cysteine, which in turn may increase production of wool. Ward, K., (1991) The Application of Transgenic Techniques for the Improvement of Domestic Animal Productivity, *Current Opinion in Biotechnology*, Vol. 2, pp. 834-839.

- 5 Furthermore, these genetically modified animals can also produce therapeutic proteins, such as insulin, growth hormone, interferon, erythropoietin, colony stimulating factor (GM-CSF), t-PA, or factor VIII, in their milk by joining the genes for these proteins with promoters from mammary specific genes such as sheep's  $\beta$ -lactoglobulin, mouse whey acid protein, or bovine  $\alpha$ S1-casein. *Id.* On the other hand, the animal's milk can  
10 also be fortified with addition of humanized proteins, such as human lactoferrin that enhance the intestinal iron absorption in infants. Lonnerdal, B. (1996) Recombinant Human Milk Proteins -- An Opportunity and a Challenge, *American Journal of Clinical Nutrition*, Vol. 63, pp. 622-626. Genetically modified pigs can even be a source for more "humanized" organs in animal to human xenotransplantation using genes such as human  
15 decay accelerating factor. Cozzi, E., et. al. (1994) Expression of Human Decay Accelerating Factor in Transgenics Pigs, *Transplantation Proceedings*, Vol. 26, pp. 1402-1403.

The articles cited above are all incorporated herein by reference.

- The following examples demonstrate that the inventor has produced a number of  
20 genetically modified animals using the sperm vector as described above. Methods in molecular genetics, flow cytometry, antibody production, hybridoma technology, in vitro fertilization, embryo manipulation, and artificial insemination used but not explicitly described in this disclosure had already been amply reported in the scientific literature. These methods are well within the ability of one skilled in the art.

25 Example I

This example illustrates the preparation of an antibody specific to sperm cells.

- Sperm cells collected from male mice were injected back into mice as antigens to immunize and produce antibodies reactive to sperm-surface antigens. Monoclonal antibodies, developed using common hybridoma techniques, were screened using flow  
30 cytometry to identify candidate antibodies that will bind to a series of different animals (mouse, pig, cow, sheep, goat, and chicken). Briefly, sperm cells were incubated with the different primary monoclonal antibodies, washed, and further incubated with a secondary antibody that specifically recognized mouse immunoglobulin. This secondary antibody, which was commercially available and well known in the art, had fluorescent molecules  
35 such as fluorescein or rhodamine conjugated to it. Once the secondary antibody molecules

were bound and washed, the flow-cytometry instrument or the FACS sorter counted the number of fluorescent sperm cells with bound primary and secondary antibodies from naked sperm cells.

Figure 2 - 7 show these flow-cytometry analyses for mAbC that bind to sperm cells of mouse, pig, cow, chicken, goat, and sheep, respectively. The Y-axis corresponds to the number of sperm cells detected while the X-axis is the relative intensity of fluorescence bound to the cell. Cross-lined peaks denote control reactions where the sperm cells were incubated only with the fluorescent anti-mouse immunoglobulin antibody. On the other hand, the shaded peaks denote the reactions where mAbC antibody and the secondary antibody were incubated with corresponding sperm cells in a mouse, pig, cow, chicken, goat, and sheep, respectively. Right shifts in the peaks denote positive binding of the mAbC antibody.

As can be seen in figure 2, greater fluorescence signals can be detected from mouse sperm cells incubated with mAbC and the fluorescent secondary antibody compared with sperm cells incubated with fluorescent secondary antibody alone. Similarly, in figure 3 and 4, greater fluorescence can be detected from pig and cow sperm cells, respectively, incubated with mAbC and the fluorescent secondary antibody as evidenced by the right shaded peaks.

In figure 5, the incubation of the fluorescence antibody alone with the chicken sperm cells did not result in any fluorescence being detected in these sperm cells. In contrast, the right peak signified fluorescence in the chicken sperm cells that have attached mAbC antibodies. Figure 5 also shows that some population of chicken sperm cells may not express the antigen recognized by mAbC as evidenced by the left shaded peak.

In figure 6, fluorescence can be detected from goat sperm cells incubated with mAbC and the fluorescent secondary antibody as evidenced by the two right shaded peaks. The left shaded peak may suggest a population of the goat sperm cells that express the antigen recognized by mAbC at a lower level than the population in the right peak. In contrast with the chicken sperm cells incubated with only the fluorescent secondary antibody in figure 5, the anti-mouse immunoglobulin fluorescent antibody seems to also bind to the goat sperm cells, but at a much reduced level than with mAbC acting as a linker.

Similarly, in figure 7, fluorescence can be detected from sheep sperm cells incubated with mAbC and the fluorescent secondary antibody as evidenced by the right shaded peaks. The distribution of the peaks again suggests the possibility that different sperm cells have different levels of the antigen recognized by mAbC.

- As seen in figures 2, 3, 4, 6, and 7, mammalian sperm cells bind, at some lower level, to the fluorescent secondary antibody. Since the secondary antibody is directed to a mouse immunoglobulin, it may have cross reactivity to other mammalian proteins on the sperm cell surfaces, which are not present in the chicken sperm cells (figure 5).  
5 Nevertheless, the shifts in fluorescence peaks upon addition of mAbC clearly demonstrate the higher affinity of the mAbC antibody to different animal sperm cells.

#### Example II

This example illustrates the ability of the monoclonal antibody mAbC to bind to DNA molecules through ionic interaction.

- 10 Different volumes of purified solutions of mAbC at a concentration of 0.5 mg/ml were added to DNA solutions containing 300ng of *Sal I* cut pCMV- $\beta$  plasmid (Figure 8, Clontech Laboratories, Inc., Cat. # 6177-1). After incubating the mixtures at room temperature for forty minutes, the mixtures were loaded on a regular one percent agarose gel and run at 20 milli-amps for one hour. Afterwards, the DNA was stained with  
15 Ethidium Bromide and visualized under UV light.

- In figure 9, lanes 1, 2, and 8 were controls with lane 1 being pure *Sal I* cut pCMV- $\beta$  plasmid and lanes 2 and 8 being *Sal I* cut pCMV- $\beta$  plasmid in Modified Tyrode's medium. Lanes 3, 4, 5, 6, and 7 corresponded to experimental reactions with the *Sal I* cut pCMV- $\beta$  plasmid incubated with 0.2 $\mu$ l, 1 $\mu$ l, 2.5 $\mu$ l, 6 $\mu$ l, and 10 $\mu$ l of mAbC at 0.5mg/ml.  
20 In lanes 5, 6, and 7, increasing amounts of DNA were retained in the wells of the gel, showing that association of the antibody, which has a positive charge, with the plasmid DNA, which has a negative charge, yielded a net electric charge of zero, resulting in a complex that no longer responds to the electric field in the gel.

#### Example III

- 25 This example illustrates the binding or coupling of the DNA to the sperm via the linker or antibody.

- DNA molecules, labeled with  $P^{32}$  using standard end labeling techniques with T4 DNA polymerase, were incubated with mouse, pig, chicken, sheep, goat, and cow sperm cells together with either mAbC, mAbD, or a control antibody specific to a *Drosophila* protein. The amount of DNA binding was measured by scintillation counting. The ratio  
30 of sperm cells to antibody were as follows:

Mouse -- 400 thousand sperm cells to 600ng of labeled DNA;  
Pig -- 600 thousand sperm cells to 800ng labeled DNA;  
Chicken -- 300 thousand sperm cells to 500ng of labeled DNA;

Sheep -- 1 million sperm cells to 500ng of labeled DNA;

Goat -- 1 million sperm cells to 500ng of labeled DNA; and

Cow -- 1 million sperm cells to 500ng of labeled DNA.

Table 1 shows that with the presence of mAbC and mAbD, sperm cells significantly bound more labeled DNA compared with reactions with no antibody or with the *Drosophila* protein-specific antibody. Reactions 1 and 2 contained only sperm cells and labeled DNA, while reactions 3 and 4 contained the *Drosophila*-protein-specific antibody together with sperm cells and labeled DNA. Reactions 5 contained mAbD while reactions 6 and 7 contained mAbC together with sperm cells and labeled DNA.

Table 1

Reactions		Mouse (cpm)	Pig (cpm)	Chicken (cpm)	Sheep (cpm)	Goat (cpm)	Cow (cpm)
1	no antibody	12471	12971	5830	15367	17749	12766
2	no antibody	15814	13713	6383	13259	16574	14398
3	Control Antibody	11541	10531	N/D	14018	155347	15351
4	Control Antibody	13653	14038	N/D	12834	15997	13918
5	mAbD	18900	16220	10314	N/D	N/D	N/D
6	mAbC	18139	16269	7294	19368	20385	20417
7	mAbC	19314	17343	9865	18437	19543	18643

N/D = Not determined

#### Example IV

This example illustrates the procedures carried out to generate genetically modified mice.

Sperm cells were collected from dissected epididymis of nine to twenty weeks old FVB male mice. Cut into small pieces, these epididymis tissues were incubated in 300μl of Modified Tyrode's medium at pH 7~8 for one hour to allow the sperm cells to escape into the medium. Once the sperm cells were collected in 300μl of medium, five micrograms of the linker antibody were added to one million sperm cells at 37°C for one hour. The sperm-linker complex was washed three times with 300μl of Modified Tyrode's medium using a standard microcentrifuge set at 3000 rpm for one and a half

minutes. The sperm-linker complex was finally resuspended in 300 $\mu$ l of medium, and one microgram of linearized pCMV- $\beta$  plasmid or a plasmid encoding for Hepatitis B surface antigen (HBsAg) was added and incubated for one hour.

To collect ovulated eggs, nine to twelve weeks FVB female mice each received an injection of 5 I.U. Pregnant Mares Serum (PMS) four days before the collection date and another 5 I.U. of human chorionic gonadotropin (hCG) two days before the collection date. Dissected ovulated eggs surrounded by cumulus cells were placed in a 35-mm petri dish containing a drop of Modified Tyrode's medium at room temperature. Afterwards, 300 $\mu$ l of sperm-linker-DNA complex prepared as described above were added directly to the ovulated eggs. The whole mix was equilibrated with CO<sub>2</sub> at 37°C with mineral oil added on top to prevent evaporation. After four hours of *in vitro* fertilization at 37°C, fertilized eggs were collected with capillary tubes and washed thrice with CZB medium. The embryos were further incubated in 300 $\mu$ l of CZB medium for 20-22 hrs before being transferred to oviducts of pseudo-pregnant female mice.

To confirm the presence of the pCMV- $\beta$  plasmid, genomic DNA isolated from embryos, ten days after transplantation into the pseudo-pregnant female mice, were analyzed by PCR using primers that detect a 480bp fragment corresponding to the CMV promoter region of the pCMV- $\beta$  plasmid (Figure 8). In figure 10, lanes 6, 7, 8, 9, 10, 12, 13, 14, 15, 17, 18, 19, 24, 33, and 40 clearly show this 480bp PCR fragment. Lanes 1 and 21 corresponded to the molecular size markers.

To confirm integration of the HBsAg plasmid into the mice genome, southern blot analysis were also performed. Genomic DNA isolated from mice's tails were digested, ran on a gel, transferred to a nylon membrane according to methods known in the art. Figure 11 shows the southern blot hybridization results with complementary probe sequences to HBsAg. Lanes 1-13 contained genomic DNA from mice born from pseudo-pregnant mice that received embryos fertilized with the sperm-linker-DNA complex described above; lanes C1-C7 contained genomic DNA from mice that were untreated or non-transgenic mice. Lanes 4, 5, and 8 show bands positive for HBsAg sequences integrated in the mice's genome, thus, demonstrating that three out the thirteen mice were genetically modified.

#### Example V

This example illustrates the procedures carried out to generate genetically modified pigs.

Ejaculated sperm cells from pigs were collected using methods generally known in the art of animal husbandry. Suspended in one milliliter of pig extender medium

(purchased from Merck, Germany, Ref.N.R.13515/0001 - dilute mixture M3 for boar sperm), fifteen million sperm cells were incubated with five micrograms of the linker antibody for forty minutes at room temperature with intermittent shaking in between. After washing the sperm-linker mixture once with pig extender medium and finally  
5 resuspending the mixture in 1.5 ml of the same medium, five micrograms of the plasmid pSEAP2-control (Figure 12, Clontech Laboratories, Inc., Cat. # 6052-1) were added and incubated with the mixture for forty minutes at room temperature. Direct injections of 200µl of the resulting sperm-linker-DNA complex into the oviducts of anesthetized female pigs resulted in fertilization *in vivo*.

10 After the pigs were born and grown to 70-day-old pigs, they were analyzed for the presence of the pSEAP2-control plasmid. Figure 13 shows the southern blot analysis of genomic DNA isolated from the tail tissues of these pigs. Briefly, genomic DNA isolated from these pigs were digested, run on a gel, and transferred to a nylon membrane according to methods well known in the art. The blot was then probed with labeled  
15 sequences from the *Not I* to *BamH I* region of the pSEAP2-control plasmid shown in Figure 12. In figure 13, M denotes the marker lanes, and 1-43 denotes the number of pigs analyzed. Hybridization signals in lanes 5, 17, 19, 25, 26, 27, 28, 30, 36, 38, 39, and 40 indicated that the pSEAP2-control plasmid had integrated into the corresponding pig's genome. In the lower right half of the figure, eight lanes with increasing copies of  
20 pSEAP2-control plasmid molecules (1, 2, 2, 4, 4, 8, 16, and 32) were also loaded on the gel together with the DNA from the experimental pigs. These eight lanes were used to estimate the copy number of pSEAP2-control plasmid integrated into the pigs based on the densitometric intensities of the bands (Figure 14). As can be seen in Figure 14, S5 had the highest intensity, which corresponds to lane 5 of figure 13.

25 In another study, secreted alkaline phosphatase (SEAP) expressed from the pSEAP2-control plasmid were also detected in 70-day old genetically modified pigs. Serum from these pigs were collected and assayed for SEAP activity using Clontech's Great EscAPE™ SEAP Chemiluminescence Detection Kit (Cat. # K2041-1) and its protocol, which is incorporated herein by reference. The SEAP enzyme expressed from  
30 Clontech's pSEAP-2 vector is thermostable. Thus, to determine the level of SEAP activity as opposed to the pigs' endogenous alkaline phosphatase enzyme activity, the assay required the deactivation of the endogenous alkaline phosphatase enzyme by heating the samples at 65°C for thirty minutes before adding the chemiluminescence substrate. As a control, figure 15 shows the result of the assay without performing this heat deactivation  
35 step. The level of total alkaline phosphatase activity was not significantly different between the genetically modified pigs and non-transgenic control pigs. In contrast, figure

16 shows the result including this heat deactivation step. Without the endogenous alkaline phosphatase activity, SEAP activity was significantly higher in the genetically modified pigs than in the non-transgenic control pigs. Thus, the pSEAP2-control plasmid had integrated well in the pigs' genome and was actively expressing the SEAP enzyme.

- 5       The preceding examples demonstrate that the inventor has produced a number of genetically modified animals using the sperm vector as described above. These data are intended only as examples and are not intended to limit the invention to these examples. It is understood that modifying the examples below does not depart from the spirit of the invention.

Claims

1. A vector for genetically modifying non-human animals or cells comprising:  
a non-human sperm cell and at least one polynucleotide molecule bound to the  
non-human sperm cell through at least one non-liposome based linker.
- 5 2. A vector of claim 1 wherein the non-liposome based linker is bound to an  
external surface of the non-human sperm cell.
3. A vector of claim 1 wherein the non-liposome based linker further  
comprises at least one polypeptide.
4. A vector of claim 3 wherein the polypeptide is a protein.
- 10 5. A vector of claim 1 wherein the non-liposome based linker is a sperm-  
specific linker.
6. A vector of claim 1 wherein the non-liposome based linker preferentially  
binds to an external surface of the non-human sperm cell.
7. A vector of claim 1 wherein the non-liposome based linker is an  
15 immunoglobulin.
8. A vector of claim 1 wherein the non-liposome based linker is an antibody.
9. A vector of claim 1 wherein the at least one polynucleotide molecule is a  
DNA molecule.
10. A vector of claim 9 wherein the DNA molecule encodes for a gene product.
- 20 11. A vector of claim 10 wherein the gene product is an RNA molecule.
12. A vector of claim 10 wherein the gene product is a protein.
13. A vector of claim 1 wherein the non-liposome based linker interacts with  
the at least one polynucleotide molecule via molecular interactions from the group



consisting of ionic interaction, covalent interaction, Vander Waals interaction, and ligand-receptor interaction.

14. A vector of claim 1 wherein the non-liposome based linker interacts with the at least one polynucleotide molecule through at least one secondary non-liposome based linker.

15. A vector of claim 2 wherein the at least one polynucleotide molecule binds to the external surface of the non-human sperm cell through the non-liposome based linker *in vivo*.

16. A non-liposome based linker for attaching at least one polynucleotide molecule to an external surface of a non-human sperm's cell comprising at least one polypeptide.

17. The non-liposome based linker of claim 16 wherein the polypeptide is a protein.

18. The non-liposome based linker of claim 16 wherein the polypeptide is an immunoglobulin.

19. The non-liposome based linker of claim 16 wherein the polypeptide is an antibody.

20. The non-liposome based linker of claim 16 wherein the at least one polypeptide preferentially binds to the external surface of the non-human sperm cell.

21. A cell derived from effecting fertilization of a non-human egg cell with a vector wherein the vector comprises:  
a non-human sperm cell and at least one polynucleotide molecule linked to the non-human sperm cell through at least one non-liposome based linker.

22. The cell of claim 21 wherein fertilization occurred *in vitro* or *in vivo*.

23. A cell of claim 21 wherein the at least one polynucleotide molecule is wholly or partially integrated into the genome of said cell.

24. A genetically modified non-human animal derived from effecting fertilization of a non-human egg cell with a vector wherein the vector comprises:

a non-human sperm cell and at least one polynucleotide molecule bound to the non-human sperm cell through at least one non-liposome based linker.

5        25. A genetically modified non-human animal of claim 24 wherein the non-human animal is a mammal.

26. A genetically modified non-human animal of claim 24 wherein the non-human animal is a bird.

10       27. A genetically modified non-human animal of claim 24 wherein the non-human animal is a pig.

28. A genetically modified non-human animal of claim 24 wherein the at least one polynucleotide molecule is wholly or partially integrated into the genome of said non-human animal.

15       29. A genetically modified non-human animal of claim 24 wherein the fertilization occurred *in vitro* or *in vivo*.

30. A genetically modified non-human animal of claim 24 wherein the at least one polynucleotide molecule binds to an external surface of the non-human sperm cell through the non-liposome based linker *in vitro* or *in vivo*.

20       31. A non-human animal that is a descendant of the genetically modified non-human animal in claim 24.

32. A cell derived from the genetically modified non-human animal in claim 25 or from a descendant of the genetically modified non-human animal in claim 24.

33. The cell of claim 32 wherein the cell is a germ cell.

25       34. A cell line derived from the genetically modified non-human animal in claim 24 or from a descendant of the genetically modified non-human animal in claim 24.

35. A method of genetically modifying a non-human animal comprising the steps:

associating at least one polynucleotide molecule to a non-human sperm cell through at least one non-liposome based linker;

- 5 effecting *in vitro* or *in vivo* fertilization of a non-human egg cell with the non-human sperm cell associated through the non-liposome based linker with the at least one polynucleotide molecule.

36. The method of claim 35 wherein the associating occurred *in vitro* or *in vivo*.

- 10 37. The method of claim 35 wherein the non-liposome based linker further comprises a polypeptide.

38. The method of claim 37 wherein the polypeptide is a protein.

39. The method of claim 37 wherein the non-liposome based linker is associated to an external surface of the non-human sperm cell.

- 15 40. The method of claim 35 wherein the non-liposome based linker is a sperm-specific linker.

41. The method of claim 35 wherein the non-liposome based linker is an immunoglobulin.

- 20 42. The method of claim 35 wherein the non-liposome based linker is an antibody.

43. The method of claim 35 wherein the at least one polynucleotide molecule is a DNA molecule.

44. The method of claim 43 wherein the DNA molecule encodes for a gene product.

- 25 45. The method of claim 44 wherein the gene product is an RNA molecule.

46. The method of claim 44 wherein the gene product is an protein.

47. The method of claim 35 wherein the non-liposome based linker interacts with the at least one polynucleotide molecule via molecular interaction from the group consisting of ionic interaction, covalent interaction, Vander Waals interaction, and ligand-receptor interaction

48. A method of generating a non-human animal that grow faster than the native non-human animal comprising the steps:  
coupling at least one polynucleotide molecule to a non-human sperm cell through at least one non-liposome based linker where the at least one polynucleotide molecule encodes for a growth hormone;  
effecting *in vitro* or *in vivo* fertilization of a non-human egg cell with the non-human sperm cell coupled through the at least one non-liposome based linker with the at least one polynucleotide molecule to form an embryo; and  
allowing the embryo to develop into an animal.

49. A method of generating disease or pathogen resistant non-human animal comprising the steps:  
coupling at least one polynucleotide molecule to a non-human sperm cell through at least one non-liposome based linker where the at least one polynucleotide molecule encodes for a gene product that confers disease or pathogenic resistance;  
effecting *in vitro* or *in vivo* fertilization of a non-human egg cell with the non-human sperm cell coupled through the at least one non-liposome based linker with the at least one polynucleotide molecule to form an embryo; and  
allowing the embryo to develop into an animal.

50. A method of producing protein comprising the steps:  
coupling at least one polynucleotide molecule to a non-human sperm cell through a non-liposome based linker where the at least one polynucleotide molecule encodes for a protein to be specifically produced in a non-human animal's milk;  
effecting *in vitro* or *in vivo* fertilization of a non-human egg cell with the non-human sperm cell coupled through the at least one non-liposome based linker with the at least one polynucleotide molecule to form an embryo;  
allowing the embryo to develop into an animal; and  
collecting the milk produced by the mature non-human animal.

51. The method of animal-human xenotransplantation comprising the steps:
- coupling at least one polynucleotide molecule to a non-human sperm cell through at least one non-liposome based linker where the at least one polynucleotide molecule encodes for a gene product;
  - 5 effecting *in vitro* or *in vivo* fertilization of a non-human egg cell with the non-human sperm cell coupled through the at least one non-liposome based linker with the at least one polynucleotide molecule to form an embryo;
  - allowing the embryo to develop into an animal; and
  - surgically removing an organ from the non-human animal for transplantation into
  - 10 humans wherein the gene product encoded by the at least one polynucleotide molecule confers tolerance in the human body after transplantation.

1/16

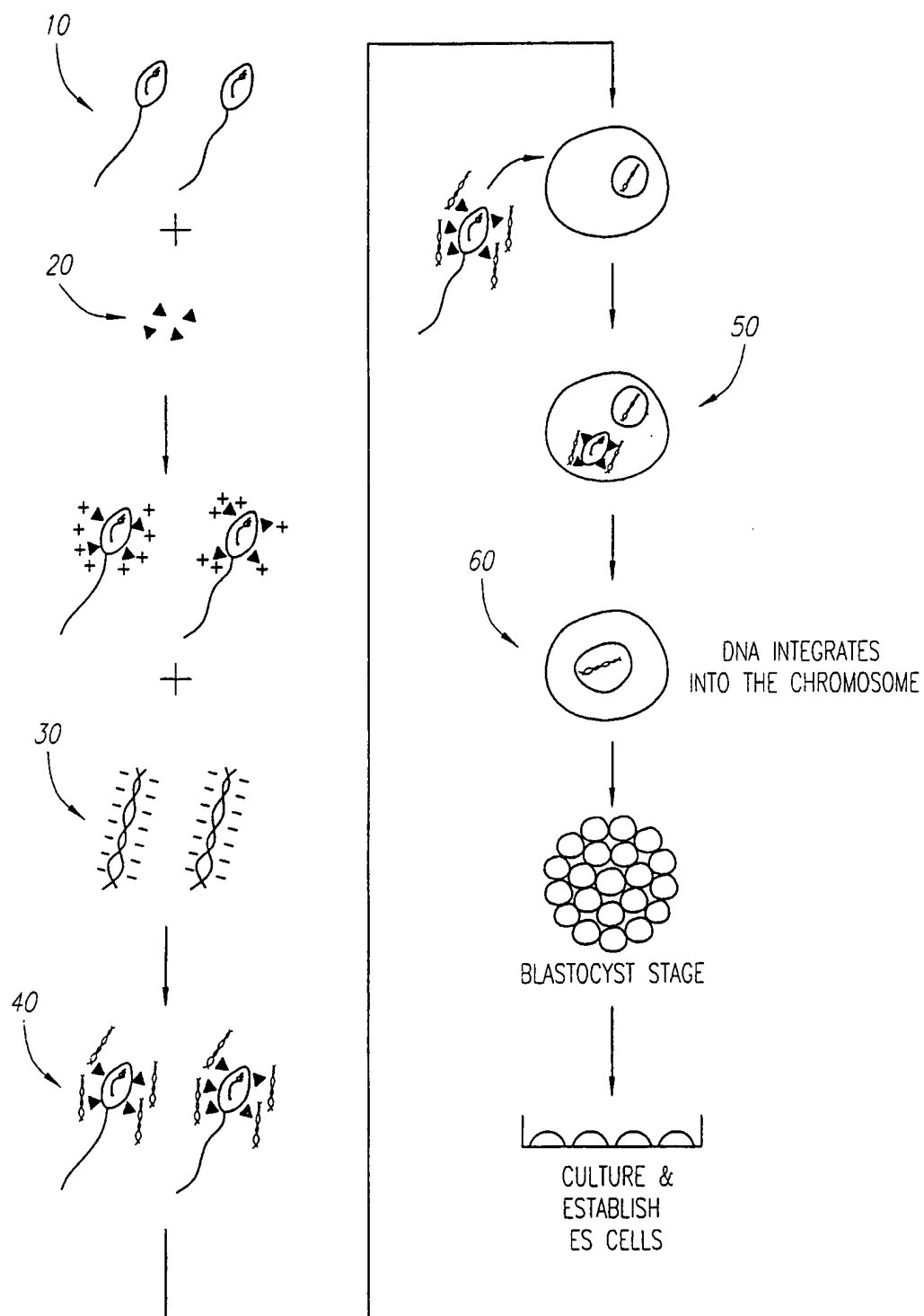


Fig.1

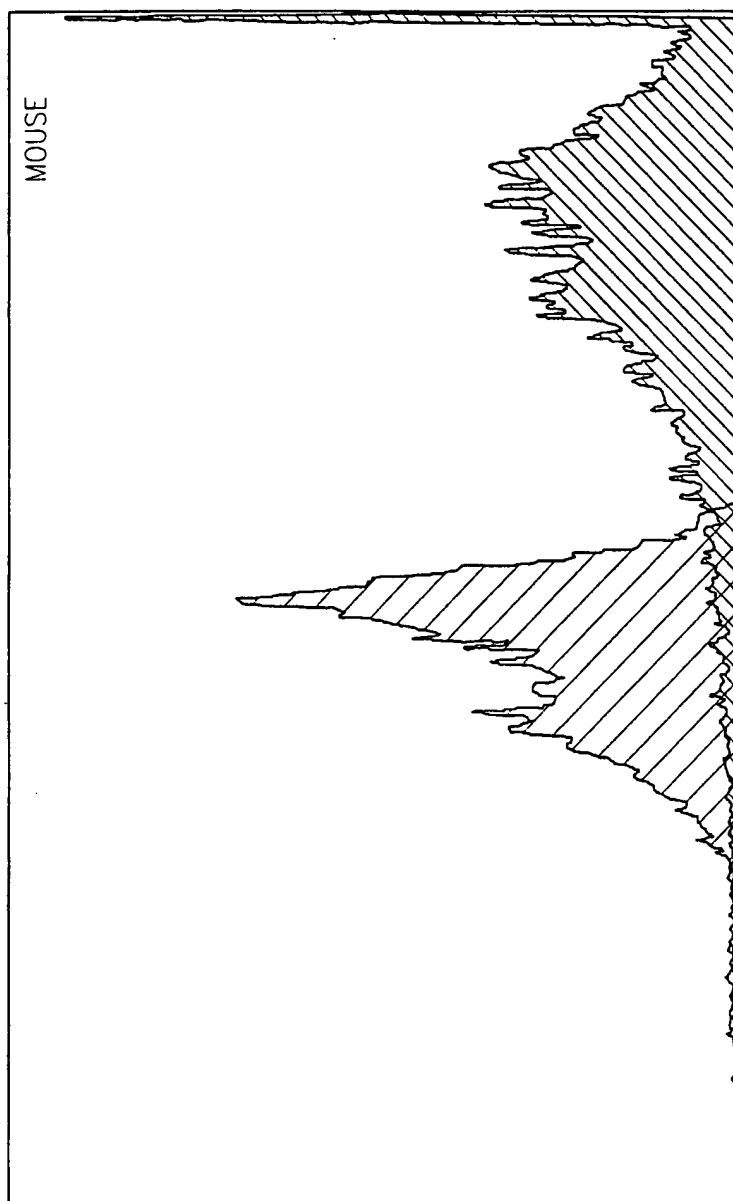


Fig. 2

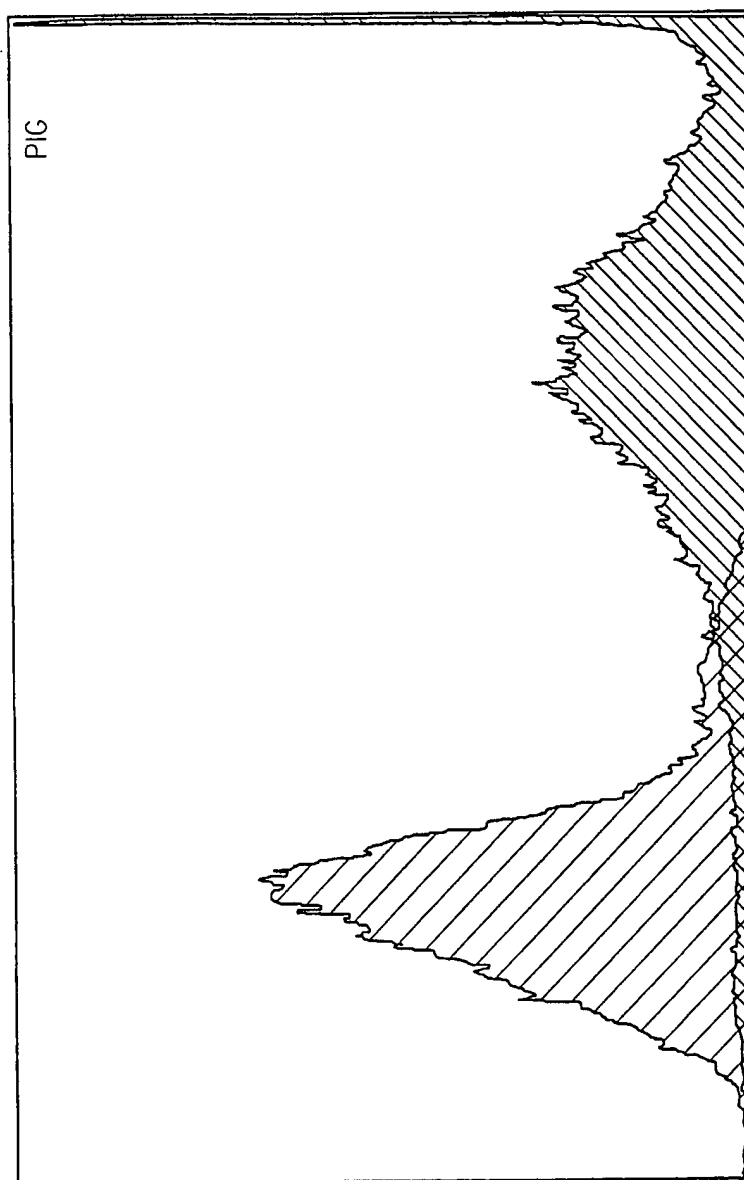


Fig. 3



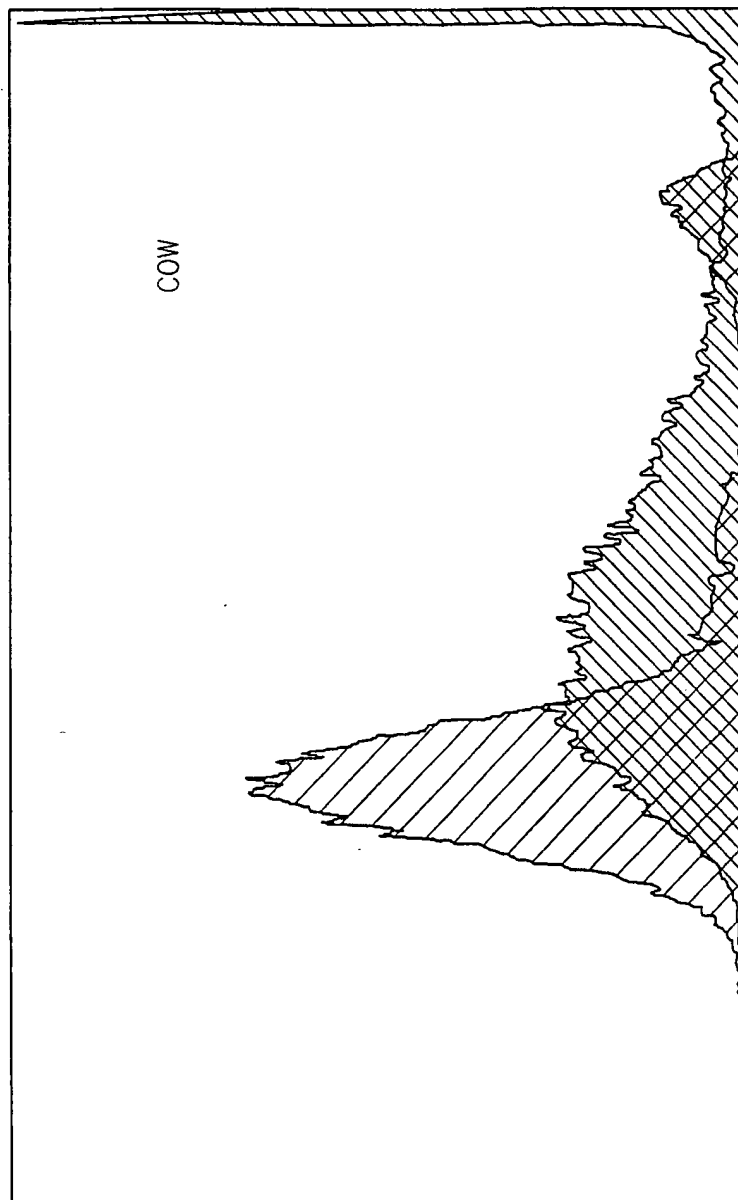


Fig. 4

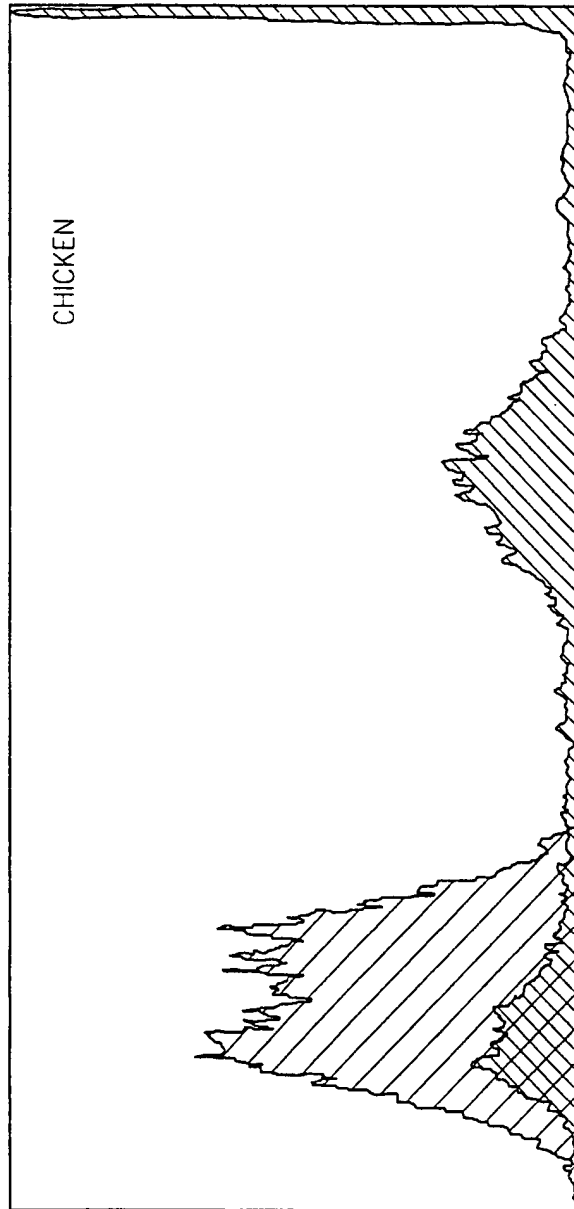


Fig. 5

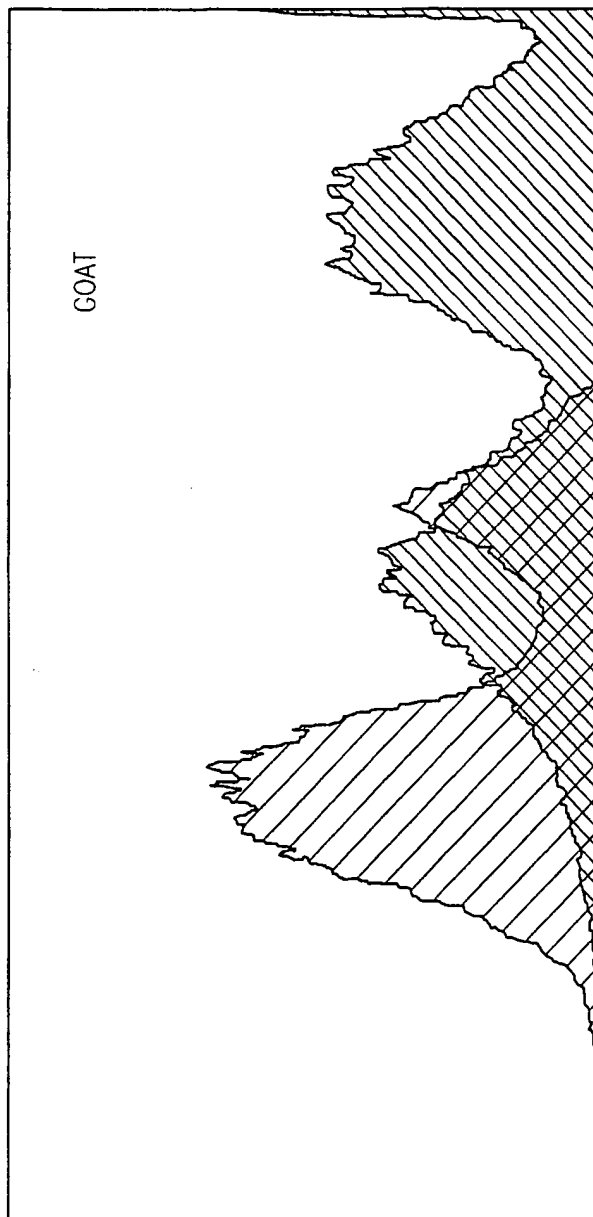


Fig. 6

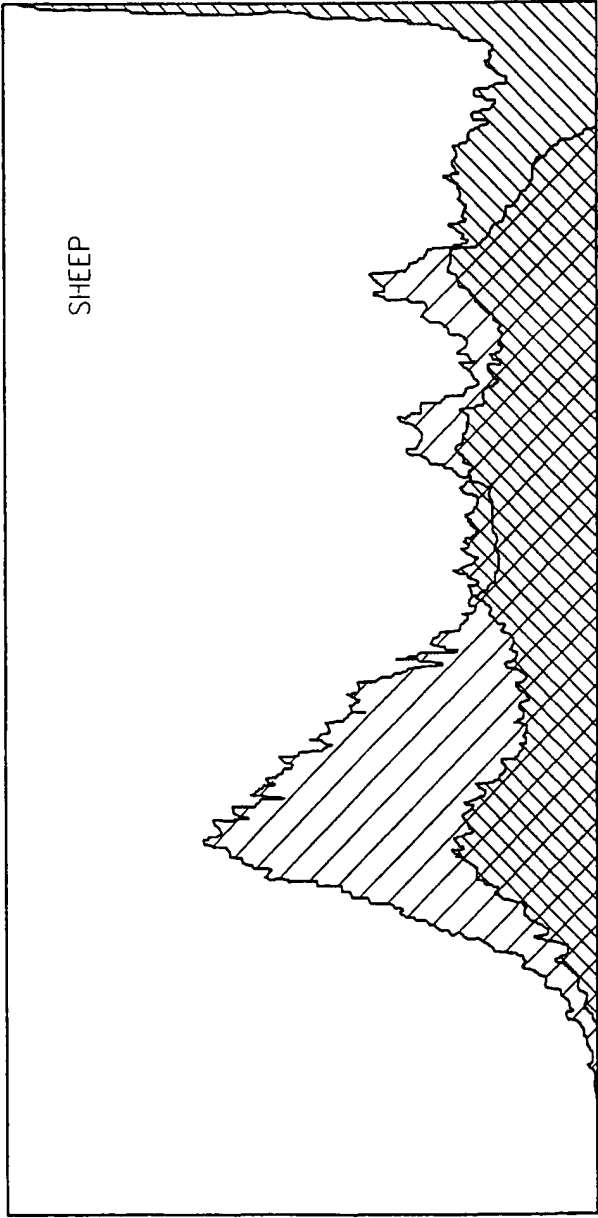


Fig. 7

8/16

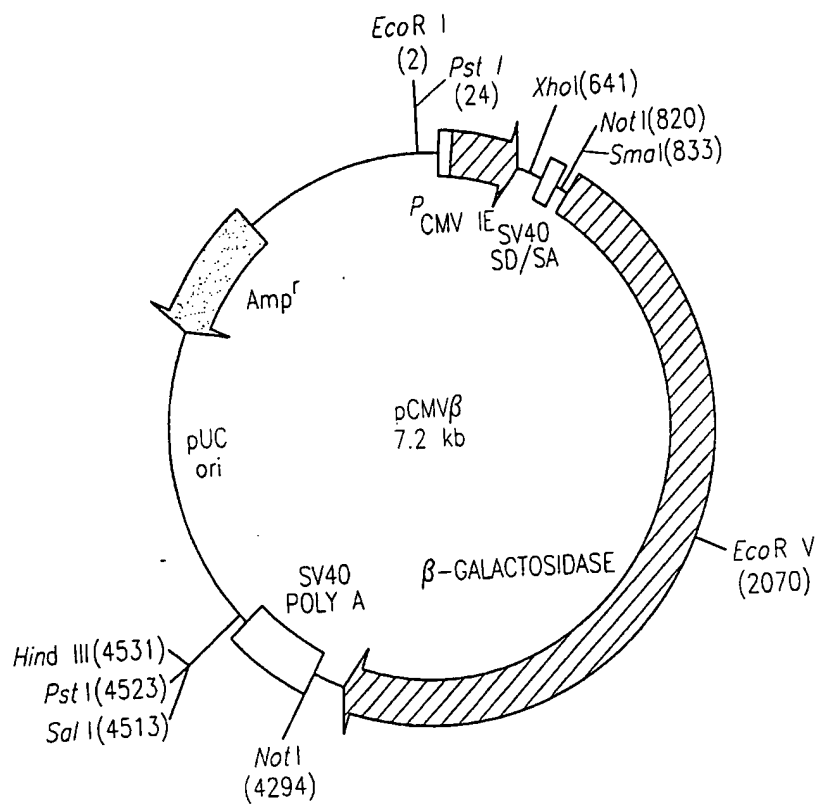
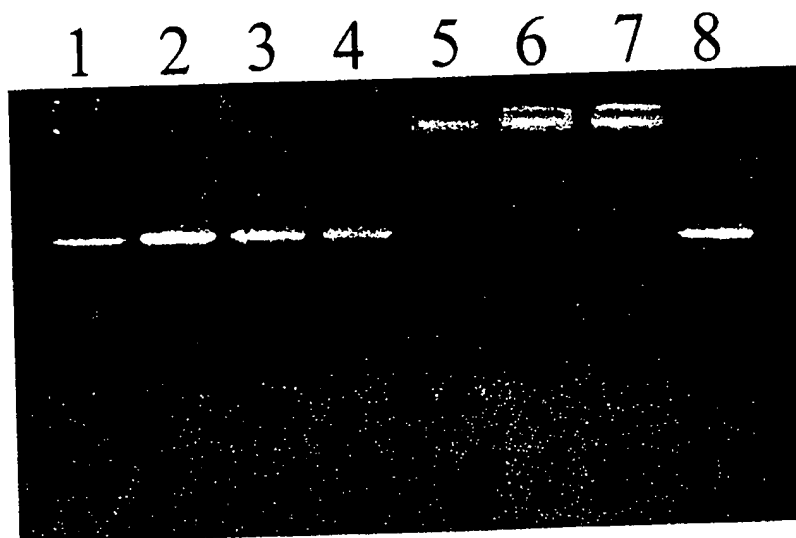


Fig. 8

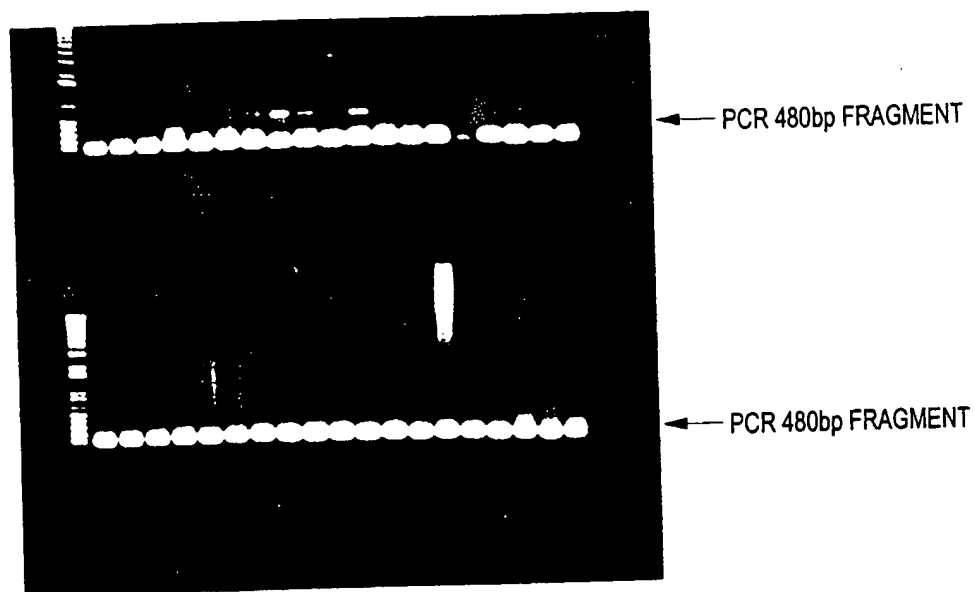
9/16



lane 1: *Sal* / cut pSEAP-2 Control DNA  
lane 2: *Sal* / cut pSEAP-2 Control DNA in Modified Tyrode's medium  
lane 3: *Sal* / cut pSEAP-2 Control DNA + 0.1  $\mu$ g mAb C  
lane 4: *Sal* / cut pSEAP-2 Control DNA + 0.3  $\mu$ g mAb C  
lane 5: *Sal* / cut pSEAP-2 Control DNA + 1.0  $\mu$ g mAb C  
lane 6: *Sal* / cut pSEAP-2 Control DNA + 3.0  $\mu$ g mAb C  
lane 7: *Sal* / cut pSEAP-2 Control DNA + 10.0  $\mu$ g mAb C  
lane 8: *Sal* / cut pSEAP-2 Control DNA in Modified Tyrode's medium

*Fig. 9*

10/16



*Fig. 10*

11/16

HBsAG SOUTHERN BLOT  
3 DAY EXPOSURE

1 2 3 4 5 6 7 8 9 10 11 12 13 C1 C2 C3 C4 C5 C6 C7



*Fig. 11*



12/16

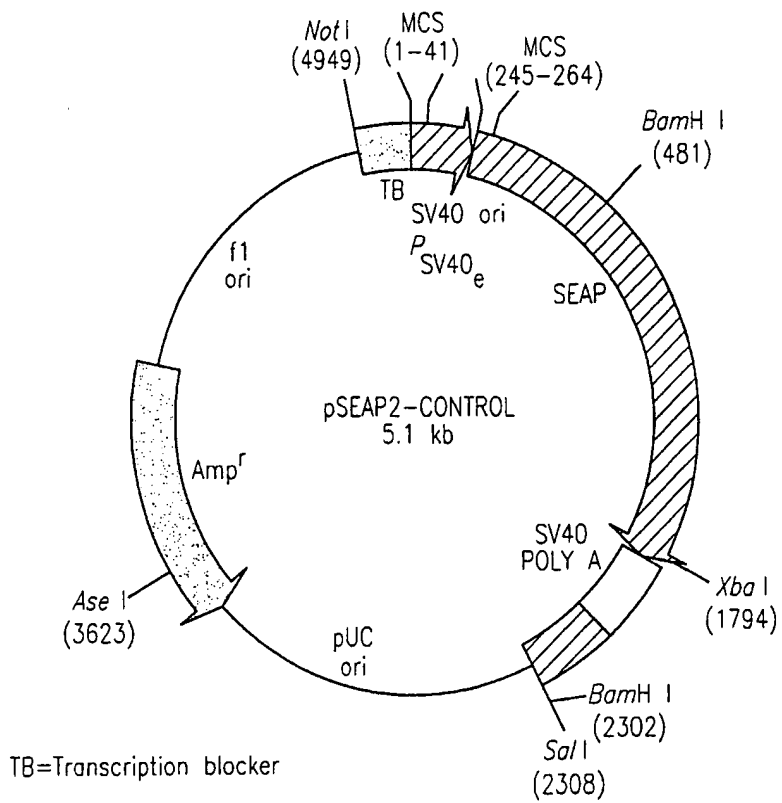
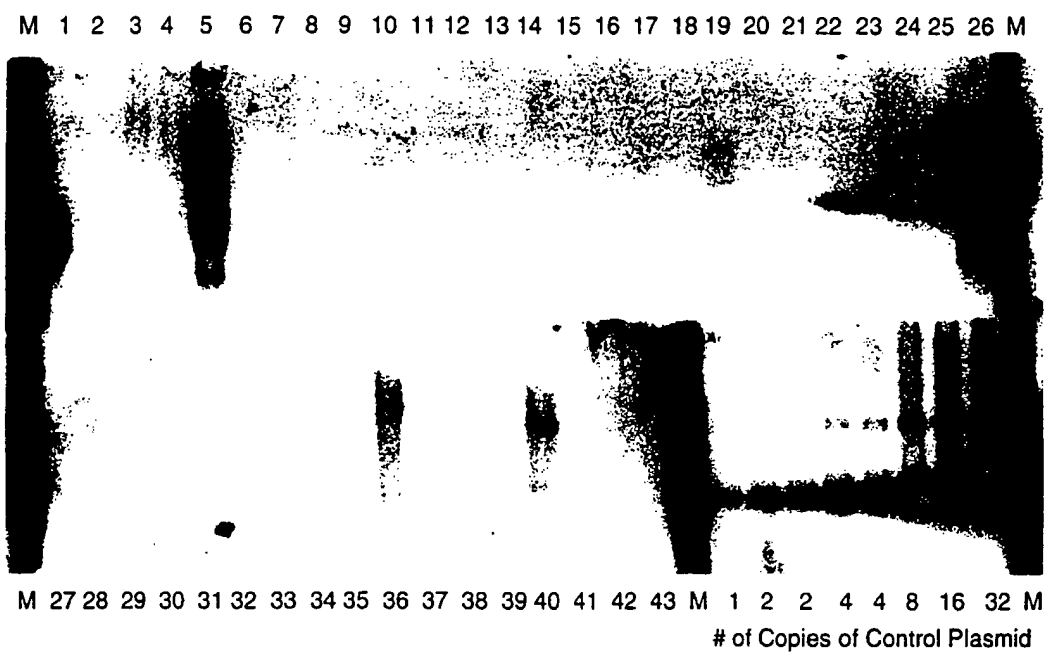


Fig. 12

13/16

## Pig Tail DNA Southern Blot

*Fig. 13*

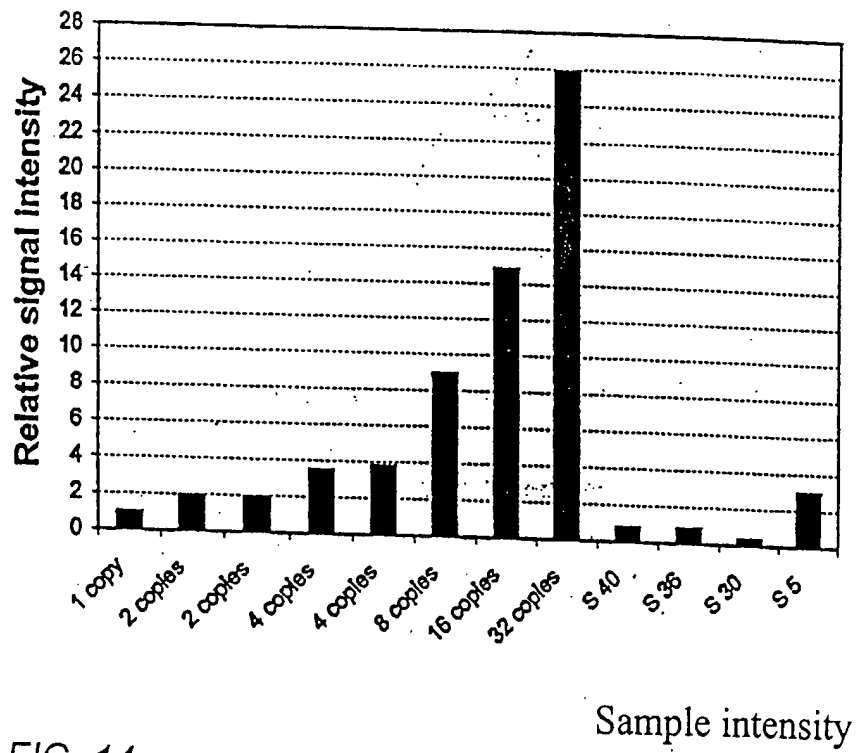


FIG. 14

Fig. 15

Secreted Alkaline Phosphatase Assay  
Non-Heated

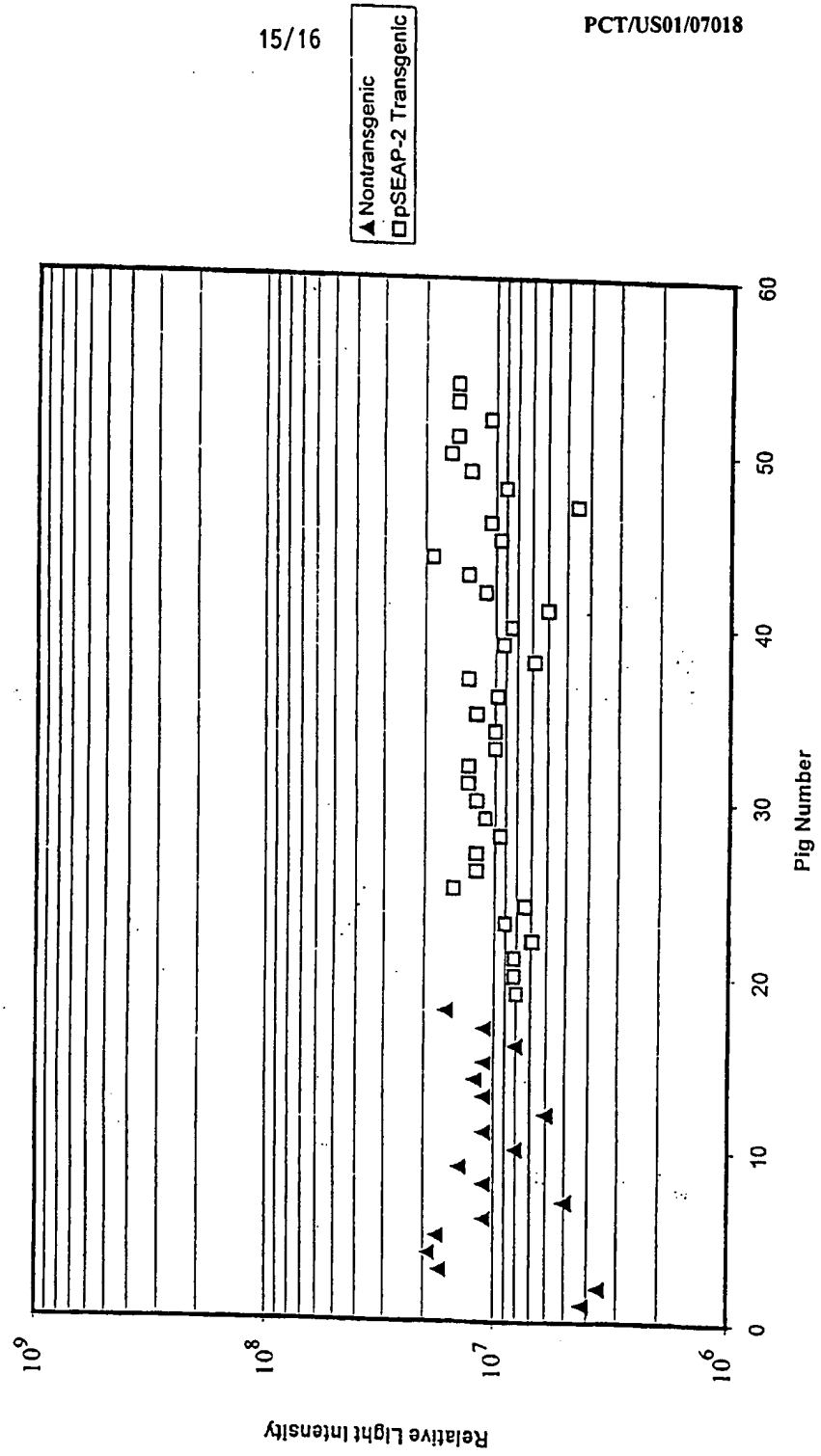


Fig. 16  
Secreted Alkaline Phosphatase Assay  
Heated

